INFECTION AND IMMUNITY, Mar. 1999, p. 1107–1115 Vol. 67, No. 3
0019-9567/99/$04.00+0

Borrelia burgdorferi Spirochetes Induce Mast Cell Activation and Cytokine Release

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Received 21 May 1998/Returned for modification 21 July 1998/Accepted 9 December 1998

The Lyme disease spirochete, *Borrelia burgdorferi*, is introduced into human hosts via tick bites. Among the cell types present in the skin which may initially contact spirochetes are mast cells. Since spirochetes are known to activate a variety of cell types in vitro, we tested whether *B. burgdorferi* spirochetes could activate mast cells. We report here that freshly isolated rat peritoneal mast cells or mouse MC/9 mast cells cultured in vitro with live or freeze-thawed *B. burgdorferi* spirochetes undergo low but detectable degranulation, as measured by [5-^{3}H] hydroxytryptamine release, and they synthesize and secrete the proinflammatory cytokine tumor necrosis factor alpha (TNF-α). In contrast to findings in previous studies, where *B. burgdorferi*-associated activity was shown to be dependent upon protein lipidation, mast cell TNF-α release was not induced by either lipidated or unlipidated recombinant OspA. This activity was additionally shown to be protease sensitive and surface expressed. Finally, comparisons of TNF-α-inducing activity in known low-, intermediate-, and high-passage *B. burgdorferi* B31 isolates demonstrated passage-dependent loss of activity, indicating that the activity is probably plasmid encoded. These findings document the presence in low-passage *B. burgdorferi* spirochetes of a novel lipidation-independent activity capable of inducing cytokine release from host cells.

Lyme disease, the most prevalent tick-associated disease in the United States, is a chronic inflammatory disorder caused by spirochetes of *Borrelia burgdorferi* sensu lato (14). Early symptoms of infection include fatigue, joint and muscle pain, and, in approximately 60% of cases, the characteristic erythema migrans lesion. If not treated, secondary pathological symptoms may manifest as arthritis, carditis, and neurologic disorders (73).

The spirochete is transmitted to the host during tick feeding and is thought to remain localized in the skin for several days (71). Thus, first contact between the spirochete and the host immune system is likely to occur in the skin. The dermal layer contains a variety of cell types, including small numbers of T cells, dendritic/Langerhans cells, keratinocytes, endothelial cells, dermal fibroblasts, and mast cells.

Mast cells can be found throughout the body but are particularly concentrated beneath the epithelial surface of the skin and mucosal layers of the genitourinary, gastrointestinal, and respiratory tracts. Mast cells release a variety of mediators in response to external stimuli. In addition to mediators such as histamine, leukotrienes, and prostaglandins, they also secrete a variety of cytokines. In mice, the production of interleukin-1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), transforming growth factor-β, and macrophage inflammatory protein 1α and 1β have been reported (13, 28, 30), and human mast cells have been shown to produce IL-4, IL-5, IL-6, IL-8, and TNF-α (8, 12, 54). While mast cells are primarily known as effector cells in allergic reactions, recent studies suggest that they can be directly activated by bacterial products and are required for the expression of immunity against certain bacteria via secretion of TNF-α, which attracts activated neutrophils to sites of infection (22, 47). Activated mast cells also appear capable of phagocytizing and killing bacterial pathogens (49), and they can present antigenic peptides to class II major histocompatibility complex (MHC)-restricted CD4$^+$ (25, 27) or class I MHC-restricted CD8$^+$ T cells (50). Thus, mast cells activated by pathogens may modulate subsequent immune or inflammatory events.

In vitro studies indicate that the Lyme disease spirochete can directly activate a variety of immune and nonimmune cell types, including macrophages, B cells, neutrophils, endothelial cells, and fibroblasts (17, 21, 45, 46, 51, 56, 65, 66, 79). Manifestations of cell activation include proliferation, cytokine and chemokine secretion, and adhesion molecule upregulation. When tested, activity was seen to be enriched in lipoprotein-containing fractions (66). Furthermore, studies with recombinant *B. burgdorferi* outer surface lipoproteins (Osps) have demonstrated that activity is dependent upon the tripalmitoyl-*S*-glyceryl-cysteine (Pam_3Cys) posttranslational lipid modification (56, 77, 79), although some investigators have detected only reduced activity in nonlipidated recombinant Osps (33).

To investigate possible interactions between *B. burgdorferi* spirochetes and mast cells, we have examined the effects of their coincubation in vitro. Here we show that mast cells incubated with either live or freeze-thawed spirochetes exhibit low-level degranulation and undergo synthesis and secretion of the proinflammatory cytokine TNF-α. We show in addition that TNF-α induction does not depend upon the Pam_3Cys lipid moiety previously implicated in the activation of cytokine release by B cells, endothelial cells, macrophages, and neutrophils (56, 77, 79). This TNF-α-inducing activity is further shown to be protease sensitive and surface expressed. Finally, comparison of *B. burgdorferi* B31 isolates with known low-, intermediate-, or high-passage histories provided evidence for passage-dependent loss of this activity.

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MATERIALS AND METHODS

***Borrelia* strains.** Low-passage *B. burgdorferi* isolates 910255 (34) and 518 (34) and high- and low-passage B31 isolates (4) were obtained from E. Hofmeister (Mayo Clinic, Rochester, Minn.). 910255 and 518 were isolated from white-

footed mice trapped in Towson, Md. (34); both strains are infectious to mice (38–40). Spirochetes were grown in BSK-II medium supplemented with 6% spirochete growth-competent rabbit serum (both from Sigma, St. Louis, Mo.) and antibiotics (rifampin and amphotericin B; both 15 μg/ml) (Sigma). Multiple aliquots of each isolate were frozen in BSK-II medium supplemented with 15% glycerol at −80°C. To obtain spirochetes for experimentation, scrapings from the frozen aliquots were inoculated into 15- or 50-ml tubes containing complete BSK-II medium and grown at 34°C for 4 to 7 days. Prior to their use in assays, the spirochetes were washed several times in (Hanks' buffered saline solution) (Sigma) by centrifugation (10,000 × *g*; 5 min), resuspended in mast cell medium, and counted by dark-field microscopy. Intermediate passages of B31 were generated by weekly subculturing of low-passage B31 in fresh complete BSK-II medium. The passages were frozen at −80°C in BSK-II medium containing 15% glycerol.

Reagents. Bacterial lipopolysaccharide (LPS) (from *Escherichia coli* O26:B6) and polymyxin B were obtained from Sigma. Purified full-length lipidated and truncated nonlipidated recombinant B31-derived OspA were gifts from John Dunn, Brookhaven National Laboratory, Long Island, N.Y. Recombinant proteins were purified by Q- and SP-Sepharose chromatography as described previously (18). Full-length lipidated recombinant OspA has a lipid modification consistent with the Pam_3Cys structure as determined by mass spectrometry (11) and has biologic properties similar to those of native OspA purified from *B. burgdorferi* (77). The truncated, nonlipidated recombinant OspA protein possesses an alanine at the amino terminus rather than a cysteine and therefore lacks the amino-terminal lipid modification (18). Lipidated OspA was solubilized and stored in 0.1% Triton X-100 in 10 mM sodium phosphate, pH 8.0. Nonlipidated OspA was stored in 10 mM sodium phosphate buffer (pH 6.0)–60 mM NaCl.

Mast cell populations. Cloned murine MC/9 mast cells (American Type Culture Collection [ATCC], Rockville, Md.) (57, 58) were grown in mast cell medium (Dulbecco's modified Eagle's medium, 10% heat-inactivated fetal bovine serum [FBS], 25 mM HEPES, 200 μM L-glutamine, 50 μg of gentamycin sulfate/ml [all Gibco-BRL, Grand Island, N.Y.] supplemented with 50% [vol/vol] IL-3-containing WEHI-3 supernatant [ATCC] ([59] grown in Iscove's modified Dulbecco's medium with 5×10^{-5} M 2-mercuptoethanol, 10% FBS, 50 μg of gentamycin sulfate/ml) at a density of 2×10^5 to 10×10^5/ml at 37°C in 5% CO_2. MC/9 cells most closely resemble mucosal-type mast cells (57). Rat peritoneal mast cells (PMCs), which are of the connective tissue type, were obtained from 6-week-old male Brown-Norway rats (Jackson Laboratories, Bar Harbor, Maine) as previously described (41). Briefly, mast cells were isolated by peritoneal lavage with 15 to 25 ml of cold, heparinized balanced salt solution and purified by centrifugation (250 × *g*; 15 min at 4°C) over a 70% (wt/vol) Percoll (Sigma) gradient. The PMCs were cultured in IL-3-containing mast cell medium. Mast cell purity, as indicated by toluidine blue staining (41), was always >95%. Rat RBL-2H3 mast cells (7, 63), which are also the mucosal type, were obtained from Bridget Wilson, University of New Mexico (UNM), and were grown in minimal essential medium with Earle's salts (without L-glutamine), 15% FBS, 200 μM L-glutamine, and 50 μg of gentamycin sulfate/ml (all from Gibco-BRL) at a density of 4 10×10^4/ml.

Mast cell degranulation. To measure mast cell degranulation, mast cells (10^6/ml) were first sensitized and labeled overnight by the addition of anti-2,4-dinitrophenol (DNP) immunoglobulin E (IgE) (44) (1 μg/ml; a gift from B. Wilson) and 2 to 4 μCi of [5-^{3}H]hydroxytryptamine ([5-^{3}H]HT) (Amersham, Arlington Heights, Il.). Following several washes with mast cell medium, ^{3}H-labeled, IgE-sensitized cells (10^5/well in triplicate) were cultured for 30 min at 37°C in 5% CO_2 with either medium alone, *B. burgdorferi* spirochetes at several spirochete-to-mast cell multiplicities, DNP-bovine serum albumin (BSA) (1 μg/ml) as a cross-linking agent, or 1% sodium dodecyl sulfate (maximum [5-^{3}H]HT release). The plates were centrifuged, 100 μl of supernatant was harvested from each well, and the amount of [5-^{3}H]HT was measured by liquid scintillation counting. The formula used to determine percent [5-^{3}H]HT release was (mean counts per minute experimental − mean counts per minute in medium alone)/(mean counts per minute maximum lysis − mean counts per minute in medium alone).

TNF-α bioassay. TNF-α released into supernatants was measured by the L929 cytotoxicity bioassay as previously described (2). Monolayer cultures of L929 fibroblasts (ATCC) were grown in 75-cm^2 flasks in complete Dulbecco's modified Eagle's medium. The L929 cells were trypsinized, plated in microwells (2.5×10^4/well), and cultured overnight at 37°C in 5% CO_2 with 50 μl of mast cell supernatant in medium containing 1 μg of actinomycin D (Sigma)/ml. The wells were then washed several times with phosphate-buffered saline (PBS) and stained with 0.5% crystal violet in 20% (vol/vol) methanol for 30 min. Following extensive washes with PBS, the incorporated dye was eluted by the addition of 50 μl of 0.1 M sodium citrate in 50% (vol/vol) ethanol (pH 4.2), and optical densities were read at 540 nm. In most experiments, the mast cells were also treated with the calcium ionophore ionomycin, (Sigma, St. Louis, Mo.) or, in later experiments, ionomycin and phorbol myristic acetate, as a positive control. TNF-α activity was determined by comparison of experimental optical densities to those obtained from a standard curve of recombinant murine TNF-α (Biological Resources Branch, National Cancer Institute, National Institutes of Health (NIH), Ft. Detrick, Md.).

Spleen cell proliferation assay. Mouse spleen cells (3×10^5/microwell) were cultured with lipidated or nonlipidated recombinant OspA in serum-free medium (HL-1; Biofluids, Bethesda, Md.) for 72 h at 37°C in 5% CO_2. Twenty-four hours prior to harvest, the wells were pulsed with 1 μCi of [^{3}H]thymidine (ICN, Irvine, Calif.). The contents of the wells were then harvested onto glass fiber filters with a Skatron cell harvester and dried, and [^{3}H]thymidine incorporation was measured by liquid scintillation counting with a Beckmann LS 1801 liquid scintillation counter.

Competitive reverse transcription (RT)-PCR. MC/9 cells (3×10^6/well in three wells) were coincubated with spirochetes (50:1) for 4 h at 37°C in 24-well plates. The well contents were harvested, and total RNA was isolated with the Qiagen RNeasy kit. Five micrograms of total RNA was then converted to single-stranded cDNA by standard protocols. To quantitate TNF-α cDNA, experimental cDNA samples were added to various amounts of the multicytokine competitor plasmid pPQRS (67) and amplified by PCR with either mouse hypoxanthine phosphoribosyltransferase (HPRT)-specific (5′-TTCCAGACAAGTTTGTTGT TGG; 3′-GCAAATCAAAAGTCTGGGGA) or TNF-α-specific (5′-CCCACGT CGTAGCAAACC; 3′-GGTTTGAGCTCAGCCCCC) primer pairs. PCR amplification was carried out in glass microcapillary tubes in a total volume of 10 μl with an Idaho Technologies model 1605 air thermocycler. By using high-efficiency hot-air heat transfer on small reaction volumes, air thermocyclers achieve high-efficiency amplification with very short cycle times (78). In preliminary experiments, we established the optimal number of cycles to achieve linear amplification of input HPRT or TNF-α cDNA. The conditions for linear amplification of HPRT were 45 cycles at 94°C for <1 s, 52°C for <1 s, and 72°C for 8 s, and for TNF-α they were 39 cycles at 94°C for <1 s, 58°C for <1 s, and 72°C for 8 s. The amplified products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. The levels of HPRT and TNF-α cDNA in samples were quantitated by titrating the competitor plasmid against the sample and determining the pPQRS concentration which gave equivalent sample and competitor bands (67). The quantification of PCR bands was aided by analysis with NIH Image version 1.61 software. Relative levels of TNF-α cDNA in the samples were determined by normalizing them to HPRT cDNA levels in the same sample.

RNase protection assay. Increased cytokine mRNA expression was detected by the RiboQuant RNase protection assay (Pharmingen, La Jolla, Calif.). MC/9 cells (3×10^6/well in three wells) coincubated with spirochetes (50:1) for 4 h at 37°C in 24-well plates were harvested, and total RNA was isolated with the Qiagen RNeasy kit. Seven to 15 μg of sample RNA was then hybridized to [α-^{32}P]UTP-labeled murine multicytokine-multichemokine RNA probe sets (mCK-1b [IL-2, -3, -4, -5, -9, -10, -13, -15, IFN-γ, L32, and GAPDH [glyceraldehyde-3-phosphate dehydrogenase]; Pharmingen) according to the manufacturer's instructions. Following RNase treatment to destroy single-stranded RNA species, "protected" cytokine RNA probes were separated on 6% denaturing acrylamide gels and visualized by autoradiography.

Crude spirochete subfractionation. Low-passage B31 spirochetes (10^8/ml) were disrupted by sonication for 5 min (90% pulse cycle; output, 10) in a water-cooled cup with a Branson model 450 sonicator. Dark-field microscopy was used to confirm disruption and loss of motility. The sonicated spirochetes were separated into soluble and insoluble material by centrifugation at 100,000 × *g* for 75 min. The 100,000 × *g* supernatant and resuspended pelleted material were then compared for induction of TNF-α release in MC/9 mast cells.

Protease treatment. Washed spirochetes (2×10^8/ml) resuspended in Hanks' buffered saline solution were disrupted by sonication (see above) and rocked gently overnight at 4°C with 0.5 U of *Streptomyces griseus* protease/ml–4% cross-linked agarose (Sigma). Spirochetes incubated overnight alone or with heat-treated (3 h at 80°C) protease-agarose beads were also included as a control. The beads were removed by settling at 1 × *g* for 10 min, and then the supernatants were assayed for TNF-α-inducing activity in MC/9 cells. Surface proteolysis of living spirochetes was carried out by a modification of a previously described procedure (16, 60). Briefly, washed live spirochetes were rocked gently for 30 min at 26°C with 0.5 U of *Tritirachium album*-derived proteinase K/ml attached to acrylic beads in PBS. Microscopic examination of treated spirochetes confirmed viability, as evidenced by normal morphology and motility. The beads were removed by settling at 1 × *g* for 10 min, and the spirochetes were washed by centrifugation, resuspended in fresh PBS, and tested for TNF-α-inducing activity. To control for retention of protease activity in the treated spirochete preparations, protease-treated medium was added to 200 U of TNF-α/ml as a control.

Statistical analysis. All experimental groups were analyzed in triplicate, and the values were imported into Excel worksheets for determination of means and standard errors and into Statview for statistical analysis. Significant differences between groups were determined by using Student's *t* test, with *P* values of <0.05 accepted as significant.

RESULTS

To determine whether *B. burgdorferi* spirochetes can activate mast cells, freshly isolated rat PMCs were incubated in vitro with *B. burgdorferi* spirochetes at a range of spirochete-to-mast cell multiplicities. As shown in Fig. 1A, for a ratio of 100:1, low but detectable degranulation occurred in these cultures, as evidenced by [5-^{3}H]HT release above background measured at

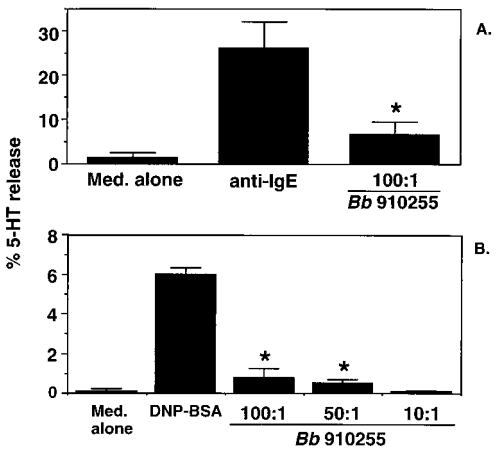

FIG. 1. Low-level degranulation of mast cells exposed in vitro to *B. burgdorferi* (*Bb*) spirochetes. [5-^{3}H]HT-labeled rat PMCs (A) or rat anti-DNP IgE-sensitized mouse MC/9 mast cells were incubated (10^5/microwell) in triplicate with various numbers of *B. burgdorferi* 910255 spirochetes, goat anti-rat IgE (1 μg/ml), DNP-BSA (1 μg/ml), or medium (Med.) alone. After 30 min, the plates were centrifuged and the supernatants were harvested and assayed for released [5-^{3}H]HT by liquid scintillation counting. The data presented are mean percent [5-^{3}H]HT release and the standard error of the mean. An asterisk indicates significant differences ($P < 0.05$) from controls, as determined by Student's *t* test.

30 min. Similar experiments were performed with cloned mouse MC/9 mast cells (57, 58), which also showed low but significant levels of degranulation in response to high doses of *B. burgdorferi* spirochetes (Fig. 1B). This low-level activation was reproducible in repeat experiments.

Because other studies had shown that *B. burgdorferi* can induce TNF-α secretion (17, 66, 77) and because mast cells have been shown to secrete TNF-α in response to bacteria (22, 47) and protozoa (9), we also tested whether *B. burgdorferi* could induce TNF-α secretion in these mast cell populations. As shown in Fig. 2, dose-dependent TNF-α production was detected at 6- and 24-h time points in PMCs and at 4- and 7-h time points, and declining by 24 h, in MC/9 mast cells. In control experiments, release of TNF-α was shown not to depend on IgE sensitization (data not shown). The delayed release of TNF-α activity (Fig. 2) suggested that *B. burgdorferi* spirochetes were inducing de novo synthesis of TNF-α. We confirmed this by demonstrating that MC/9 mast cells incubated with spirochetes in the presence of actinomycin D, an inhibitor of RNA synthesis, did not secrete TNF-α (Fig. 3). We next used competitive RT-PCR (67) to determine the levels of TNF-α mRNA in MC/9 mast cells exposed to *B. burgdorferi* spirochetes. As shown in Fig. 4, mast cells stimulated with *B. burgdorferi* spirochetes for 4 h showed a 10-fold increase in TNF-α mRNA over background compared to the 16-fold increase observed in MC/9 cells stimulated with the calcium ionophore ionomycin.

RNase protection assays were used to determine whether additional mast cell cytokine genes are transcribed following contact with *B. burgdorferi* spirochetes or recombinant OspA proteins. MC/9 cells activated by ionomycin and PMA for 4 h showed upregulated expression of various cytokine genes, including IL-3, IL-4, IL-5, IL-9, IL-10, IL-13, and IL-15 genes. However, no increases in mRNA levels for any of these genes were observed following 4 h of exposure to *B. burgdorferi* spirochetes or recombinant lipidated or nonlipidated OspA. Constitutive expression of IL-9 and IL-13 mRNAs, however, were observed in all samples (data not shown). MC/9 cells activated in vitro with calcium ionophore A23187 and PMA have been previously reported to secrete IL-2, IL-3, IL-4, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (32).

To determine whether mast cell activation could be induced by other *B. burgdorferi* isolates, we tested two others, B31 (4) and 518 (34), for their ability to induce TNF-α production in MC/9 mast cells. Although *B. burgdorferi* spirochetes do not possess classical endotoxin (74), mast cells can be activated by bacterial LPS (42, 62). To exclude the possibility that activation by these spirochetes was due to contaminating bacterial LPS (46, 66), we also tested the capacity of spirochetes to induce TNF-α release in the presence of polymyxin B, which binds and inactivates LPS (35). As shown in Fig. 5, all three strains of *B. burgdorferi* tested activated TNF-α release, but TNF-α release was not inhibited by polymyxin B. Furthermore, LPS was unable to induce TNF-α secretion in MC/9 cells, despite inducing potent B cell proliferation in mouse spleen cells, which was inhibitable by polymyxin B (data not shown).

Because *B. burgdorferi* lipoproteins have been shown to be potent activators of a variety of cell types (33, 45, 46, 51, 65, 66), we tested whether purified recombinant lipidated or nonlipidated OspA was capable of eliciting TNF-α release from mast cells. Both forms were tested because in other systems where *B. burgdorferi* lipoproteins were found to be active, their activity was dependent upon the Pam_3Cys lipid modification (56, 77, 79). However, neither the lipidated nor the nonlipidated OspA was capable of inducing TNF-α secretion by MC/9 mast cells when tested at a range of concentrations (Fig. 6B). Lipidated OspA clearly retained mitogenic activity for other cell types, as demonstrated by its ability to induce dose-dependent B cell proliferation (Fig. 6A). These results indicate that the Pam_3Cys moiety is not responsible for *B. burgdorferi* spirochete-mediated TNF-α induction in mast cells.

To further characterize this *B. burgdorferi*-associated TNF-α-inducing activity, we disrupted *B. burgdorferi* spirochetes by sonication and subjected the resulting extracts to ultracentrifugation (100,000 × *g*; 75 min) in order to separate soluble (supernatant) from insoluble (pelleted) material. As shown in Fig. 7, mast cell TNF-α-inducing activity in sonicated spirochete extracts was found to be exclusively associated with the insoluble fraction.

Since live spirochetes were clearly capable of inducing TNF-α release, and this activity appeared not to involve soluble factors or secreted components (Fig. 7), we considered it likely that spirochete surface components might be involved. To determine whether TNF-α-inducing activity was dependent upon protein, we treated spirochete extracts overnight at 4°C with immobilized protease, removed the protease, and then tested the treated spirochetes for their ability to elicit TNF-α secretion in MC/9 mast cells. As shown in Fig. 8A, whereas untreated spirochete extracts or extracts treated with heat-treated protease retained TNF-α-inducing activity, extracts treated with active protease lost all activity. To control for possible residual protease activity in the protease-treated spirochete preparations, we incubated the protease-treated supernatants with TNF-α but detected no loss of activity. To determine whether spirochete-associated TNF-α-inducing activity is surface expressed, living spirochetes were subjected to limited nonlethal surface proteolysis (60) and then tested for their ability to induce TNF-α secretion. As shown in Fig. 8B, live spirochetes treated with proteinase K lost the ability to induce TNF-α secretion, whereas untreated spirochetes or spirochetes treated with heat-treated proteinase K retained activity. As before, no residual protease activity was detected in protease-treated supernatants. Thus, TNF-α-inducing activity is surface expressed and is sensitive to protease treatment.

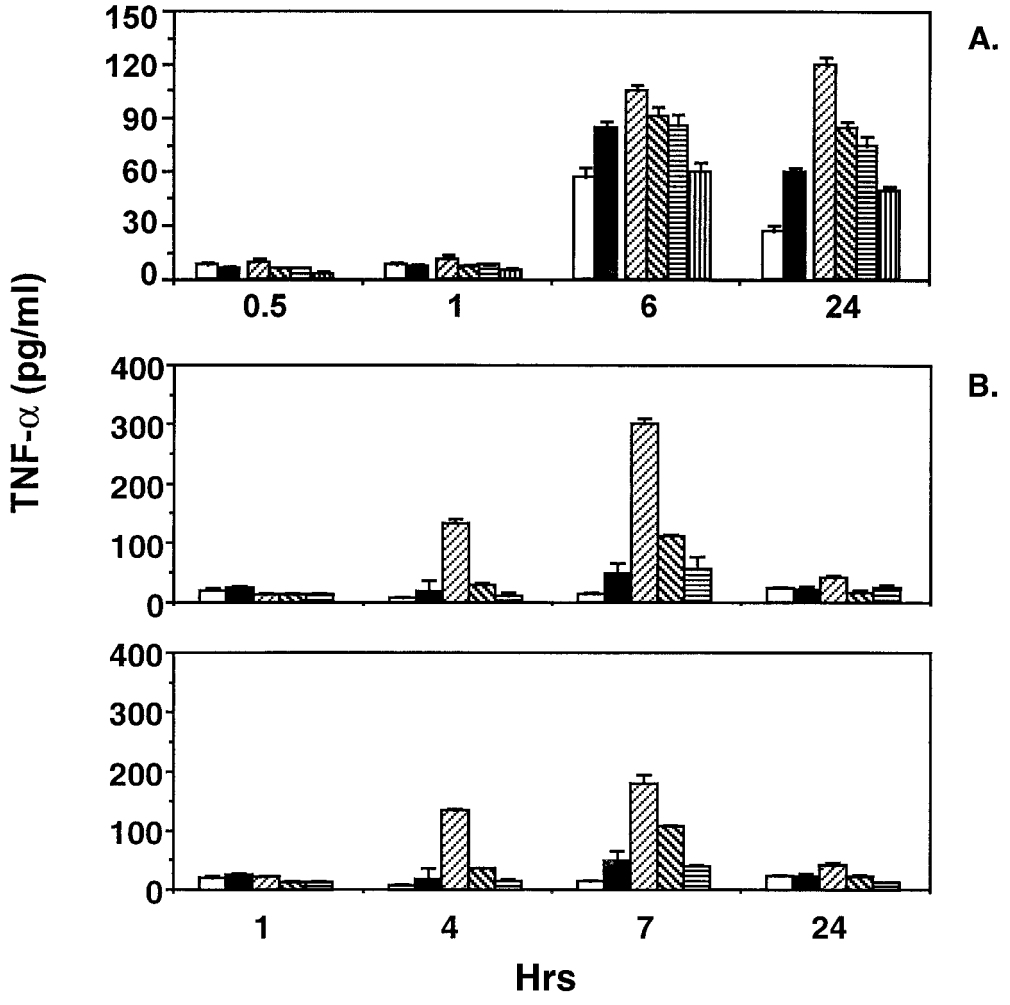

FIG. 2. TNF-α release by mast cells following in vitro exposure to *B. burgdorferi* (*Bb*) spirochetes. Rat PMCs were exposed to live spirochetes (A) and mouse MC/9 mast cells were exposed to live (upper panel) or freeze-thawed (lower panel) spirochetes (B) in triplicate at multiplicities of 100:1 (▨), 30:1 (▧), 10:1, (▤), or 3:1 (▥) spirochetes/cell. Goat anti-rat IgE (1 μg/ml) or DNP-BSA (1 μg/ml) as cross-linking agents (■) or medium alone (□) were also used. After various times, the cell supernatants were collected and frozen at −80°C. TNF-α in the supernatants was then determined by using the L929 bioassay, as described in Materials and Methods. The data presented are the means and standard errors of the means of triplicate determinations. The data are representative of >3 experiments with similar results.

Prior studies have shown that *B. burgdorferi* infectivity is attenuated by in vitro passage (61). To determine whether in vitro passage influences the expression of TNF-α-inducing activity, we subjected low-passage B31 spirochetes to weekly in vitro passage in BSK-II medium and then tested spirochetes from different passages along with low- and high-passage B31 isolates for their ability to induce TNF-α release from mouse MC/9 mast cells. As shown in Fig. 9, compared to that of low-passage B31 isolates, TNF-α-inducing activity was significantly diminished after five passages and completely lost by seven passages.

DISCUSSION

Previous studies have documented the ability of *B. burgdorferi* spirochetes or their products to induce a variety of effects, including cell proliferation, cytokine and chemokine production, increased phagocytosis, upregulation of adhesion molecules, and nitric oxide production, in a range of cell types, including macrophages, endothelial cells, neutrophils, B cells, and fibroblasts (21, 45, 46, 56, 65, 66, 77, 79). Because mast cells occur in high numbers in skin (~10^4/mm) (76) and since infecting *B. burgdorferi* spirochetes are known to stay localized in the skin for several days (71), mast cells are likely to be among the first host cells which contact spirochetes.

In this study, we show that *B. burgdorferi* spirochetes have the ability to induce degranulation and TNF-α release from mouse MC/9 mast cells and rat PMCs in vitro. Although the levels of degranulation observed were low and were only evident at high spirochete-to-mast cell multiplicities (e.g., 100:1), they were statistically significant in multiple experiments. Dose-dependent TNF-α release from stimulated mouse MC/9 mast cells and rat PMCs was observed, with maximal release occurring at >4 h postchallenge (Fig. 2), preceded by a 10-fold increase above background in synthesis of TNF-α mRNA detected at 4 h (Fig. 4). The delayed kinetics of TNF-α release by mast cells suggested that exposure to spirochetes induces de novo synthesis and secretion of this cytokine, and this was confirmed in studies which demonstrated complete inhibition of TNF-α release by the transcription inhibitor actinomycin D (Fig. 3).

Our failure to detect activation of other inducible cytokine genes in MC/9 mast cells stimulated with *B. burgdorferi* spiro-

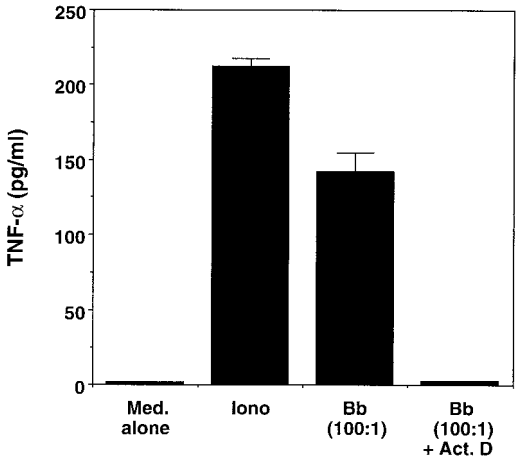

FIG. 3. Mast cell TNF-α secretion induced by *B. burgdorferi* (*Bb*) spirochetes is inhibited by actinomycin D. MC/9 mast cells (10^5/well) were incubated with medium (Med.) alone, ionomycin (Iono) (1 μM), or freeze-thawed *B. burgdorferi* spirochetes (100:1) for 7 h at 37°C and 5% CO_2 in the presence or absence of actinomycin (Act.) D (10 μg/ml) (Sigma) as previously described (30). The supernatants were removed, and TNF-α was measured as described in the legend to Fig. 2. The data are representative of two experiments with similar results.

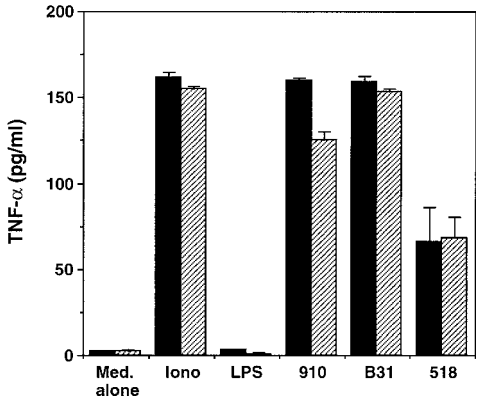

FIG. 5. Mast cell TNF-α-inducing activity is present in other *B. burgdorferi* isolates and is not due to LPS contamination. MC/9 mast cells were incubated with medium (Med.) alone, ionomycin (Iono) (1 μM), LPS (20 μg/ml), or spirochetes from several different *B. burgdorferi*, isolates in the presence (▨) or absence (■) of polymyxin B (20 μg/ml) at 37°C and 5% CO_2. After 8 h, the supernatants were collected and frozen at −80°C. TNF-α in the supernatants was measured as described in the legend to Fig. 2. The data are representative of two experiments with similar results.

chetes suggests that TNF-α induction is fairly specific. While ionomycin and PMA stimulation of MC/9 cells led to increased expression of IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, IL-13, and IL-15 mRNAs at 4 h, incubation with *B. burgdorferi* failed to enhance mRNA levels for any of these cytokines (data not shown). IL-9 and IL-13 mRNAs were found to be constitutively expressed in MC/9 cells. Interestingly, the range of mediators released by activated mast cells appears to depend on the nature of the stimulus (1). For example, IgE receptor-mediated activation, which occurs when specific antigen is encountered, typically leads to rapid exocytosis of preformed granules containing histamine, heparin, and serine proteases followed by slower release of additional mediators, such as cytokines, leukotrienes, and prostaglandins, which are synthe-

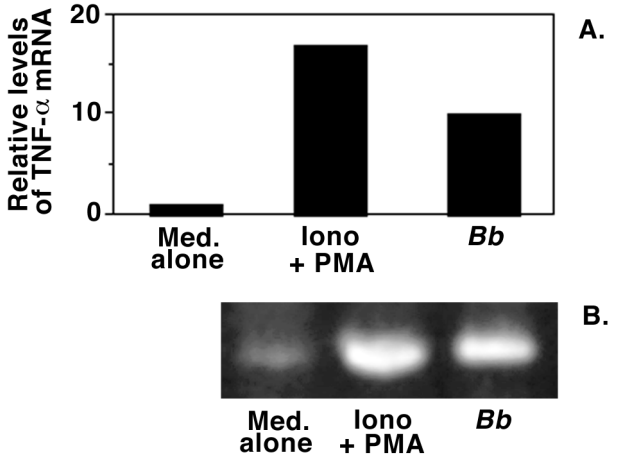

FIG. 4. Mast cell TNF-α mRNA synthesis is induced by *B. burgdorferi*. (A) Relative TNF-α mRNA levels as measured by competitive RT-PCR. Total RNA from MC/9 mast cells exposed for 4 h in vitro at 37°C to either medium (Med.) alone, *B. burgdorferi* (*Bb*) (100:1), or ionomycin (Iono) (1 μM) and phorbol myristic acetate (PMA) (50 μg/ml) was subjected to first-strand cDNA synthesis and then amplified in the presence of the multicompetitor plasmid pPQRS (67) with HPRT- and TNF-α-specific primer pairs. TNF-α cDNA was quantitated as described in Materials and Methods. (B) TNF-α mRNA levels in HPRT-normalized samples as detected by RT-PCR. cDNA samples prepared as described above were normalized to an equivalent input HPRT cDNA concentration by competitive RT-PCR with pPQRS and then subjected to amplification with TNF-α-specific primer pairs. The amplified products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide.

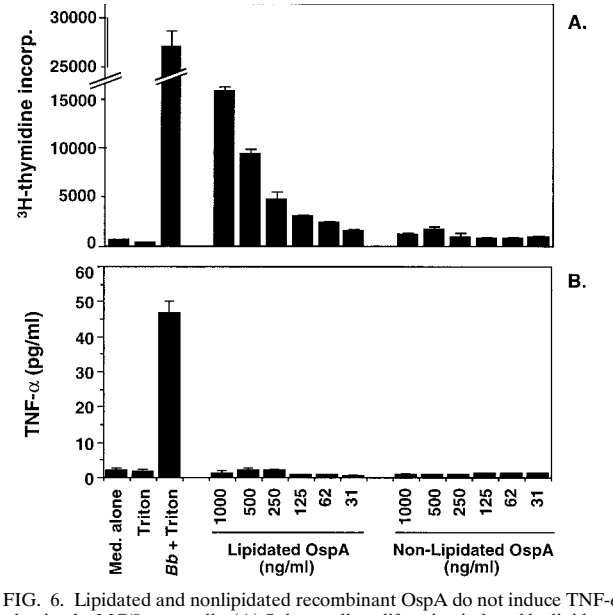

FIG. 6. Lipidated and nonlipidated recombinant OspA do not induce TNF-α production by MC/9 mast cells. (A) Spleen cell proliferation induced by lipidated OspA. Naive mouse spleen cells (3×10^5/microwell) were incubated with either medium (Med.) alone, medium alone containing 0.001% Triton X-100, *B. burgdorferi* (*Bb*) spirochetes (100:1) in medium containing 0.001% Triton X-100, or serial twofold dilutions of purified recombinant lipidated or unlipidated OspA for 72 h at 37°C in 5% CO_2. Twenty-four hours prior to harvest, the wells were pulsed with 1 μCi of [^{3}H]thymidine. The well contents were harvested onto glass fiber filters and counted by liquid scintillation counting. The data presented are the mean cpm and standard errors of the mean for triplicate cultures. (B) TNF-α production in MC/9 mast cells. MC/9 mast cells were incubated with either medium alone, medium alone containing 0.001% Triton X-100, *B. burgdorferi* spirochetes (100:1) in medium containing 0.001% Triton X-100, or different concentrations of purified recombinant lipidated or unlipidated OspA for 72 h at 37°C in 5% CO_2 diluted in MC/9 medium. After 8 h, the supernatants were harvested and TNF-α was measured as described in the legend to Fig. 2. The data are representative of three experiments with similar results.

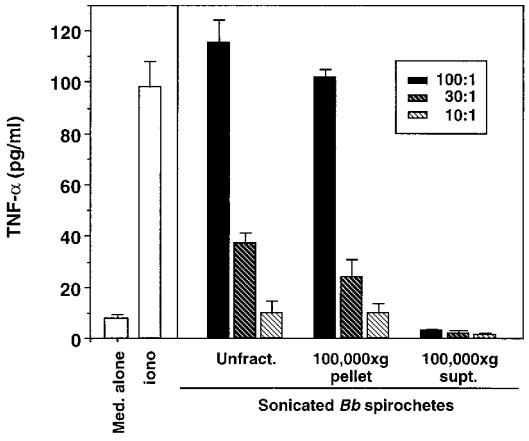

FIG. 7. Mast cell TNF-α-inducing activity is associated with the nonsoluble fraction in sonicated *B. burgdorferi* (*Bb*) extracts. Sonicated B31 spirochetes (10^8/ml) were centrifuged for 75 min at 100,000 × *g*, and the supernatant (supt.) and resuspended pelleted fractions were tested for TNF-α-inducing activity in MC/9 cells at 100 spirochete equivalents per mast cell. Ionomycin (iono) (1 μM) was included as a positive control. After 8 h, the supernatants were collected and frozen at −80°C until being assayed. TNF-α in the supernatants was measured as described in the legend to Fig. 2. The data are representative of two experiments with similar results. Unfract., unfractionated.

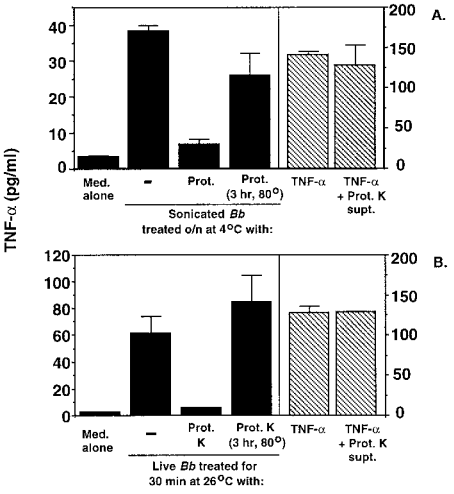

FIG. 8. *B. burgdorferi* (*Bb*)-associated TNF-α-inducing activity is sensitive to protease treatment and is surface expressed. (A) Sonicated *B. burgdorferi* spirochetes (10^8/ml) were incubated overnight (o/n) at 4°C with either 0.5 U of immobilized protease/ml (Prot.) covalently attached to 4% agarose beads, 0.5 U of heat-treated (3 h at 80°C) immobilized protease/ml, or medium (Med.) alone (−). After removal of the protease beads by gravity sedimentation, the spirochetes (100:1) were incubated with MC/9 mast cells for 8 h and the supernatants were harvested and tested for TNF-α as described in the legend to Fig. 2. Protease beads were also incubated with medium alone, removed by gravity sedimentation, and incubated with recombinant TNF-α (125 μg/ml) to control for the presence of residual protease activity (hatched bars). The data presented are representative of three experiments with similar results. (B) Live spirochetes (2×10^8/ml) were incubated with either 0.5 U of immobilized proteinase K (Prot. K)/ml attached to acrylic beads, 0.5 U of heat-treated immobilized proteinase K/ml attached to acrylic beads, or medium alone for 30 min at 26°C as previously described (6, 60). The treated spirochetes remained viable, as evidenced by their continued motility. The beads were then removed by gravity sedimentation, and the spirochetes were tested for TNF-α induction in MC/9 cells as described in the legend to Fig. 2. Protease beads were also incubated with medium alone, removed by gravity sedimentation, and incubated with recombinant TNF-α (125 μg/ml) to control for the presence of residual protease activity (hatched bars). The data presented are representative of two experiments with similar results. The error bars represent standard errors of the mean.

sized de novo (29). In contrast, exposure of rat PMC to bacterial cholera toxin failed to stimulate release of preformed mediators but induced expression of IL-6 while inhibiting production of TNF-α (43). In addition, bacterial endotoxin was found to stimulate IL-6 production without inducing granule exocytosis (42).

The chemical nature and localization of this TNF-α-inducing activity in the spirochete were also investigated. Previous studies have established that spirochete lipoproteins are potent inducers of cell activation (45, 46, 65, 66, 77) and that their activity depends upon the Pam_3Cys posttranslational lipid modification (21, 56, 65, 77, 79). Our studies, however, point to the existence of a lipidation-independent pathway of cell activation, since neither lipidated nor nonlipidated recombinant OspA induced TNF-α release in MC/9 cells (Fig. 6B). This failure to activate mast cells was not due to inactive lipoprotein, since the same preparation of lipidated OspA induced dose-dependent B-cell proliferation (Fig. 6A). While these experimental results do not rule out the participation of lipoproteins in mast cell activation, they do indicate that the lipid modification is not a requirement for expression of this activity. Recent experiments indicate that CD14, the surface receptor on neutrophils and monocytes/macrophages which binds to and facilitates signaling by LPS (75, 81) and other lipidated bacterial products (15, 68), is also the signaling receptor for *B. burgdorferi* lipoproteins (80). MC/9 mast cells, which do not respond to LPS (Fig. 5), probably do not express CD14 and therefore are unable to bind and be activated by the Pam_3Cys moiety of *B. burgdorferi* lipoproteins, such as OspA (Fig. 7). This mast cell TNF-α-inducing activity was additionally found to be sensitive to protease treatment (Fig. 8A) and appears to be expressed on the spirochete surface, as evidenced by loss of activity in live, intact spirochetes subjected to short-term proteolysis (60) (Fig. 8B). The protease sensitivity of this activity distinguishes it from *B. burgdorferi* lipoprotein-mediated activity, which was found to be relatively protease insensitive (66). The probable association of this activity with the spirochete membrane is also supported by the finding that all of the activity partitioned to the insoluble, pelleted fraction (100,000 × *g*; 75 min) following sonication (Fig. 7). The finding that *B. burgdorferi* organisms can activate mast cells in vitro is consistent with previous studies, which found that a variety of microbial products, including bacterial hemolysins, toxins, and LPSs, can directly (1, 9, 43, 51, 69) or indirectly (24) activate mast cells. In fact, recent studies reported that recombinant lipidated OspA of *B. burgdorferi* upregulated CD28 expression in stem cell factor-derived bone marrow mast cells (51).

The actual surface-expressed molecule(s) responsible for TNF-α induction and the mechanism by which this molecule activates mast cells remain to be determined. Since the Pam_3Cys lipid moiety itself does not appear to be involved (Fig. 6), it is likely that a surface-expressed polypeptide domain of a bacterial lipoprotein or integral membrane protein interacts directly with mast cell surface receptors. The only well-studied example of direct activation of mast cells by bacterial products is the mannose-binding lectin FimH protein of enterobacteria, which activates mast cells by binding to an unknown mannose-containing receptor on the surface of mast cells (48). The failure of the rat basophilic leukemia cell line RBL-2H3 to be activated by exposure to *B. burgdorferi* spirochetes (data not shown) indicates likely heterogeneity of responsiveness among different mast cell populations, which may reflect differences in their expressions of the relevant receptor. As for spirochete candidate proteins, the spirochete outer membrane contains ~20 to 25 unique proteins with molecular masses ranging from

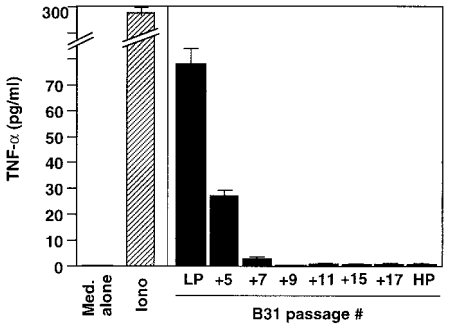

FIG. 9. In vitro passage of *B. burgdorferi* isolate B31 leads to loss of mast cell TNF-α-inducing activity. The abilities of low-passage *B. burgdorferi* B31 (<10 in vitro passages) (LP), high-passage B31 (>50?) (HP), and multiple-intermediate-passage B31 substrains derived from low-passage B31 by weekly subinoculation into fresh BSK-II medium (+5, +7, +9, +11, +15, and +17) to induce TNF-α secretion by MC/9 mast cells were compared. The 8-h supernatants were assayed for TNF-α as described in the legend to Fig. 1. The data are representative of three experiments with similar results. The error bars represent standard errors of the means.

20 to 60 kDa, comprising both membrane-spanning polypeptides and hydrophilic polypeptides anchored by N-terminal lipids (i.e., lipoproteins) (10). This activity is apparently plasmid encoded, since low-passage *B. burgdorferi* strains express activity (Fig. 5) but the activity is rapidly lost during in vitro passage (Fig. 9). In vitro passage of *B. burgdorferi* leads to loss of linear and circular plasmids encoding a variety of proteins (5, 60, 61, 70, 72) and to loss of infectivity (36, 55, 70, 72). We are currently comparing plasmid profiles in active and nonactive B31 clones in an attempt to identify the plasmid which encodes this activity. Preliminary studies with B31-derived mutant strains lacking Osp have provided evidence that Osps A through D are not involved (74a).

Mast cells activated by spirochetes in vivo probably participate in subsequent immune and/or inflammatory events. For example, early TNF-α production by spirochete-activated dermal mast cells would help initiate local inflammation, attracting inflammatory cells into sites of spirochete replication in the skin (2, 20). Such early inflammatory responses would lead eventually to the generation of T- and B-cell-dependent acquired immune responses, which control spirochete replication. In addition to promoting inflammation via TNF-α secretion, mast cells may also directly contribute to immune clearance of spirochetes (53). Mast cells activated by the mannose-specific FimH protein of enterobacteria have been shown to be capable of phagocytizing and killing bacteria (49). In addition, activated mast cells can present antigen to class II-restricted $CD4^+$ (25, 26) and class I-restricted $CD8^+$ (50) T-cell clones and hybridomas. Antigen-presenting function by mast cells appears to at least partially depend upon cytokines, with GM-CSF and IL-4 upregulating antigen presentation and IFN-γ downregulating it (27). In support of a possible protective role for mast cells in vivo, $WBB6_{F1}$ mast cell-deficient mice infected with *B. burgdorferi* spirochetes develop higher levels of joint swelling and have decreased capacity to resolve their joint swelling compared to normal $WBB6_{F1}$ mice (37a). On the other hand, mast cells are prominent in the synovia of patients with Lyme arthritis (19, 64), suggesting that they may also contribute to disease manifestations, perhaps via TNF-α secretion. It has been hypothesized that direct activation of host cells by *B. burgdorferi* spirochetes and release of proinflammatory mediators, such as IL-1β (45), IL-6 (46), IL-8 (21), and TNF-α (17, 45, 46, 65, 66, 77), play contributory roles in the pathogenesis of Lyme disease. Consistent with this scenario, type 1 cytokine responses, which are proinflammatory, also positively correlate with disease severity in mice and humans (3, 23, 31, 37, 39, 52, 82). Levels of TNF-α are elevated in the sera and synovial fluids of patients with Lyme disease and the sera of experimentally infected mice (17). Other sources of TNF-α in vivo are monocytes and macrophages, which have been shown to secrete TNF-α in vitro following exposure either to whole spirochetes (36) or to purified native or recombinant *B. burgdorferi* lipoproteins (45, 46, 65, 66, 77).

While this novel spirochete-associated, lipidation-independent TNF-α-inducing activity was identified by using mast cells, the same activity may be capable of activating nonmast cells as well. Proof of this will require testing of purified native or recombinant protein. Despite much evidence favoring a requirement for Pam_3Cys in *B. burgdorferi* lipoprotein-mediated host cell activation, one recent study found that nonlipidated OspA was able to elicit cytokine release from human monocytes, although the cytokine levels were severalfold lower than those elicited by lipidated OspA (33), suggesting that the Pam_3Cys moiety provides an adjuvant effect but may not be strictly required. Thus, the relative importance of lipidation-independent and Pam_3Cys-dependent cytokine-inducing activity in *B. burgdorferi* remains to be clarified.

In summary, we report the partial characterization of a *B. burgdorferi* spirochete-associated activity which induces mast cells to undergo low-level degranulation and secretion of the proinflammatory cytokine TNF-α. The activity is protease sensitive, surface expressed, and, in contrast to the major bioactivity previously associated with *B. burgdorferi* (21, 56, 65, 77, 79), does not require lipidation. The activity is lost during in vitro passage of *B. burgdorferi* spirochetes and is therefore probably plasmid encoded (5, 36, 55, 60, 61, 70, 72). The possible influence of this activity on spirochete infectivity and pathogenicity is currently being tested.

ACKNOWLEDGMENTS

This work was supported by a Howard Hughes Interim Funding Award from the University of New Mexico and a grant from NIH (AI-33397) to S.P.N.

We thank E. Hofmeister for the *B. burgdorferi* strains, John Dunn for recombinant OspA, Bridget Wilson for the mast cell reagents, and Cherese Bellino for technical assistance.

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Editor: J. R. McGhee